RelB nuclear translocation regulates B cell MHC molecule, CD40 expression, and antigen-presenting cell function

Brendan J. O'Sullivan, Kelli P. A. MacDonald, Allison R. Pettit, and Ranjeny Thomas*

Centre for Immunology and Cancer Research, University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland, 4102, Australia

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Mice with targeted RelB mutations demonstrated an essential role for RelB in immune responses and in myeloid dendritic cell differentiation. Human studies suggested a more global transcriptional role in antigen presentation. Burkitt lymphoma cell lines were used as a model to examine the role of RelB in antigen presentation. After transient transfection of BJAB with RelB, strong nuclear expression of RelB-p50 heterodimers was associated with increased APC function and expression of CD40 and MHC class I. Antisense RelB in DG75 reduced antigen-presenting capacity and CD40-mediated up-regulation of MHC molecules. The data indicate that RelB transcriptional activity directly affects antigen presentation and CD40 synthesis. Stimulation of RelB transcriptional activity may provide a positive feedback loop for facilitating productive APC/T cell interactions.

RelB is a member of the NF-κB/Rel family of proteins that includes p50, p52, RelA (p65), c-Rel, and RelB (1). RelB is present in the cytosol, bound to p50 or p52 (2, 3) and an inhibitory IκB protein forming an inactive trimeric complex. RelB can be rapidly mobilized to the nucleus after IκB degradation, a process potentiated by cell surface-signaling events, including CD40 ligation (4). Although RelB encodes a potent transcription-activating domain, few genes have been shown to be directly regulated by RelB. Potential candidates include TNFα, MHC class I, and the Igκ locus (5–7).

Recent evidence suggested that RelB may regulate the differentiation of myeloid dendritic cells (DC) for effective antigen presentation (5, 8, 9). Thus, mice with a targeted RelB mutation have an atrophic thymic medulla and absent lymph nodes, and lack differentiated myeloid DC in the spleen (8, 10, 11). Peripheral immature DC, such as Langerhans cells, are present in normal numbers, but immune responses, including delayed type hypersensitivity, are impaired. However, in normal human blood and lymph nodes, nuclear localization of RelB was found to be associated with antigen-presenting cells (APC), including differentiated DC, follicular DC, and some activated B cells. From these studies, we hypothesized that RelB may play a more general transcriptional role in APC function than one restricted to DC differentiation (12).

Therefore, the current studies were undertaken to examine whether RelB nuclear location would directly influence APC function and relevant MHC and costimulatory molecules. For this purpose, several Burkitt lymphoma (BL) cell lines, with varying levels of antigen-presenting capacity were used. In these BL cell lines, up-regulation of APC function associated with increased cell surface CD40 and MHC class I after overexpression of RelB, and down-regulation of APC function after introduction of antisense RelB are demonstrated. The data provide evidence that specific transcriptional activity of RelB can directly affect antigen presentation through several key molecules.

Materials and Methods

Cell Lines and Culture. All cell culture was carried out in medium RPMI 1640 (GIBCO) supplemented with 10% FCS (CSL, Parkville, Australia), 300 μg/ml L-glutamine, 10 mg/ml gentamicin (DBL, Mulgrave, Australia), and 200 units/ml penicillin (CSL). Epstein–Barr virus (EBV)-negative cell lines BJAB, BL30, and DG75 have been previously described (13, 14). EBV-transformed B cells [lymphoblastoid cell line (LCL)] were prepared from peripheral blood of normal donors as previously described (15). Monocyte-derived DC (MDDC) were prepared from adherent peripheral blood mononuclear cells, cultured for 4 days in medium supplemented with 800 units/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 400 units/ml recombinant human IL-4 (both from Schering Plough) as previously described (16). The human cytolytic T lymphocyte (CTL) line, LC13, is HLA B8 restricted and specific for the EBV nuclear antigen 3 (EBNA3) mapped to the minimal epitope FLRGRAYGL (17). BL30 (EBO-pLPP-Sig-FLRG) and BL30 (EBO-pLPP-FLRG) are stably transfected with plasmids encoding the same EBNA3 peptide sequence FLRGRAYGL (residues 337–347), with or without a signal sequence encoded by the adenovirus E3 protein (18). To induce nuclear translocation of RelB, 5×10^6 BL cells were incubated for 24 h in the presence of 50 ng/ml soluble CD40 ligand (sCD40L) (provided by Immunex).

Plasmids and Transfection of Cell Lines. A full-length RelB cDNA was generated by PCR amplification of LCL cDNA. Expression constructs pRelB and pAntiRelB were generated by cloning sense and antisense RelB cDNA into *Eco*RI and *Not*I sites of pCDNA3 (Invitrogen). p50 and p52 expression plasmids were constructed by cloning full-length murine p50 and p52 cDNAs (both obtained from R. Bravo, Princeton, NJ) into the *Bam*HI site of pCDNA3. pGreenLantern (GIBCO) constitutively expresses GFP. For transfection, 10 μg pRelB and pCDNA3 or 5 μg pGreenLantern were added per 10^7 cells, and cells were electroporated (960 μF, 200 V) in a total volume of 250 μl medium. Transfected cells were incubated for 24 h in a total volume of 3 ml in a 6-well plate before use. Stable antisense cell lines were generated by transfection of DG75 with pAntiRelB and then selection in 1.5 mg/ml G418 (GIBCO). For determination of nuclear and cytoplasmic location of RelB, 10^5 cells were cytospun, fixed, and stained for RelB by using immunoperoxidase and diaminobenzidine staining as described (19).

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Abbreviations: APC, antigen-presenting cell; BL, Burkitt lymphoma; CTL, cytolytic T lymphocyte; DC, dendritic cell; EBV, Epstein–Barr virus; EMSA, electrophoretic mobility-shift assay, GFP green fluorescent protein; LCL, lymphoblastoid cell line; MDDC, monocyte-derived dendritic cell; MLR, mixed leucocyte reaction; TNF, tumor necrosis factor; EBNA3, EBV nuclear antigen 3; sCD40L, soluble CD40 ligand; hIFN-γ, human IFN-γ; PE, phycoerythrin.

*To whom reprint requests should be addressed. E-mail: rthomas@medicine.pa.uq.edu.au.

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Flow Cytometry and Cell Sorting. Twenty-four hours after transfection, cells were stained with either mouse anti-human CD40 (G28.5, a gift from P. Lipsky, Dallas, TX), MHC class I [W632, American Type Culture Collection (ATCC)], HLA-DR (DK22, Dako), CD80 (Ancell, Bayport, MN), CD86 (Ancell), or CD54 (BI-RR-0001, a gift from P. Lipsky), followed by biotinylated rabbit anti-mouse Ig (Dako) and then streptavidin-phycoerythrin (PE) (Dako) as previously described (20). Cells were analyzed by using a Coulter Epics Elite flow cytometer. For some experiments, cells transfected 24 h previously with pGreenLantern were sorted as green fluorescent protein $(GFP)^+$ cells by using the same instrument.

Electrophoretic Mobility Shift Assays (EMSA). Nuclear extracts were prepared as previously described (12), and protein estimations used a Protein Assay kit (Bio-Rad). EMSA were carried out as previously described (12) by using 10 μg protein and 2 μg of either anti-RelB, anti-p50, or anti-p52 (all from Santa Cruz Biotechnology) as indicated, per lane.

Assays of APC Function. All APC were treated with mitomycin C (Sigma) as previously described (16). For the mixed leucocyte reaction (MLR), allogeneic T cells were purified from nylon wool nonadherent peripheral blood mononuclear cells derived from healthy donors by negative selection with anti-CD19, anti-CD16, anti-CD56, anti-HLA-DR, and goat anti-mouse Ig-conjugated magnetic beads (MACS, Miltenyi Biotec, Auburn, CA) as previously described (12). Varying numbers of APC were incubated with 10^5 allogeneic T cells for 5 days. [^{3}H]Thymidine (1 μCi/well, ICN) was added for the last 18 h. Plates were harvested by using an automated harvester and counted by using a Packard TopCount NXT. Results are expressed as mean $\pm$ SEM of triplicate wells. For assays of MHC class I-restricted peptide presentation to the CTL line LC13, production of human IFN-γ (hIFN-γ) in the supernatant was measured 24 h after incubation of APC and LC13. hIFN-γ was measured by ELISA by using a Quantiferon-γ kit (CSL) according to the manufacturer's instructions.

Immunoprecipitations. BJAB (4×10^7) were transfected with pRelB or pCDNA3 by electroporation and incubated for 24 h in 10 ml medium. Nuclear and cytoplasmic extracts were prepared as described for EMSA, immunoprecipitated with 1 μg/ml of anti-p50, and immunoblotted for RelB. For blocking, p50 peptide (Santa Cruz) was added at a 5-fold concentration over anti-p50 and incubated for 1 h before immunoprecipitation.

Statistical Analysis. The mean fluorescent intensity (MFI) of expressed proteins was determined in three separate experiments by flow cytometry. Differences were analyzed by using paired t tests. $P < 0.05$ was considered significant.

Results

Nuclear RelB Translocation in Human BL Cell Lines. MDDC and LCL that have translocated RelB to the nucleus are efficient APC in MLR (12). Initial experiments characterized RelB location and APC function of BL cell lines that are readily amenable to genetic manipulation. The EBV-negative BL cell lines BJAB and BL30 were less effective stimulators of allogeneic T cells in MLR than MDDC or LCL (Fig. 1*A*). The data suggest that, unlike DC and LCL, the BL cell lines may lack constitutive RelB heterodimers in the nucleus. By using a specific NF-κB-labeled sequence, EMSA was performed by using BJAB nuclear extracts. Two major bands corresponding to p50 homodimers (Fig. 1*B*, lower band) and p50-c-Rel/RelA/RelB heterodimers (Fig. 1*B*, upper band) were observed. Because RelB is able to form heterodimers only with p50 or p52 (2, 3), these antibodies were employed for supershift experiments. RelB (Fig. 1*B*, lane 1) and p52 (Fig. 1*B*, lane 3) were not present constitutively in nuclear extracts from BJAB, but low levels of p50 were detected (Fig. 1*B*, lane 2).

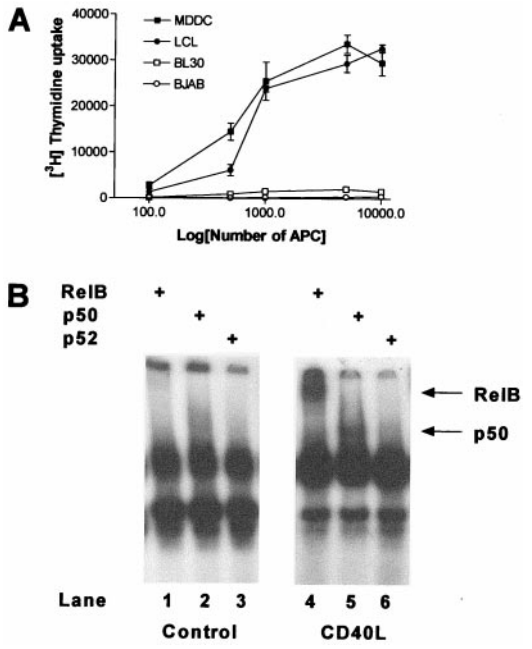

Fig. 1. APC function and RelB location in BL cell lines. (*A*) Varying numbers of either MDDC, EBV-transformed lymphocytes (LCL), or the BL cell lines BJAB or BL30 were incubated with 10^5 purified allogeneic T cells for 5 days, and T cell proliferation was measured by incorporation of [^{3}H]thymidine. Data are the mean of triplicate wells $\pm$ SD and are representative of three separate experiments. (*B*) Nuclear extracts were prepared from BJAB after incubation for 24 h in the presence or absence of 50 ng/ml sCD40L. For supershift assays, 10 μg of nuclear extract was incubated with either 2 μg of RelB, p50, or p52 antibody for 1 h at 4°C, followed by labeled NF-κB probe, and separated on a 4% polyacrylamide gel. No supershift was demonstrable in the presence of rabbit Ig control (not shown).

The lack of constitutive nuclear RelB in BJAB was confirmed by immunohistochemical staining and immunoblotting. RelB was present in the cytoplasm but not the nucleus of the cells (data not shown). CD40L has been shown to induce nuclear translocation of RelB in primary B cells and B cell lines (4). Therefore, to induce RelB translocation, BJAB were cultured with soluble CD40L for 24 h, and nuclear extracts were separated by EMSA (Fig. 1*B*). Nuclear extracts derived from sCD40L-treated BJAB contained RelB (Fig. 1*B*, lane 4) and p50 (Fig. 1*B*, lane 5) but not p52 (Fig. 1*B*, lane 6). Data obtained for another BL cell line, BL30, were comparable to those for BJAB (not shown). These data demonstrate constitutive low level nuclear p50, and induction of RelB and p50 nuclear translocation by CD40 ligation in BJAB and BL30.

RelB Overexpression Enhances Antigen-Presenting Capacity of BL Lines. To examine directly whether nuclear RelB location, and thus transcriptional activity, was associated with antigen-presenting capacity, RelB was overexpressed in BJAB and BL30 cell lines (see Supplementary Fig. 6 at www.pnas.org). Initial experiments examined the cellular location of expressed RelB. BJAB were transfected with RelB or pCDNA3, and NF-κB complexes from nuclear extracts were analyzed by EMSA and supershift assays (Fig. 2*A*). Although not constitutive, RelB (Fig. 2*A*, lane 2) and p50 (Fig. 2*A*, lane 3) were detected in pCDNA3-transfected BJAB nuclear extracts, most likely as a result of electroporation and DNA introduction. However, larger

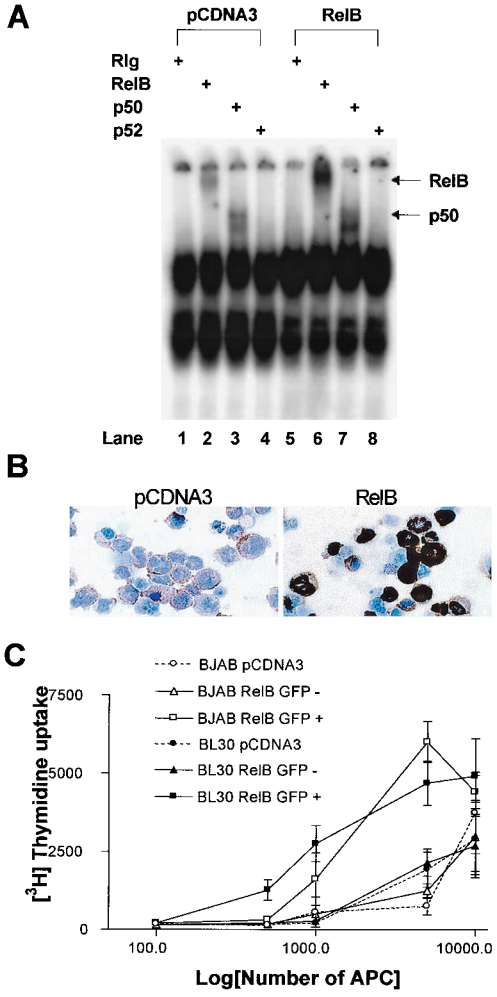

Fig. 2. Overexpression of RelB enhances APC function of BL cell lines. (*A*) BJAB were transfected with either RelB or pCDNA3 and incubated for 24 h before preparation of nuclear extracts, incubation with antibody and labeled NF-κB probe, and separation as in Fig. 1*B*. (B) BJAB were transfected with either RelB or pCDNA3, together with the GFP-expressing plasmid pGreenLantern, and incubated for 24 h; then, GFP$^+$ cells were sorted by flow cytometery. An amount equal to 10^5 of each population of GFP$^+$ cells was cytospun, fixed, and stained for RelB by using an immunoperoxidase technique. RelB is stained with diaminobenzidine (brown), and the nucleus is counterstained with hematoxylin (blue) at magnification ×130. (*C*). BJAB and BL30 cell lines were transfected, and GFP$^+$ and GFP$^-$ cells were sorted and incubated with allogeneic T cells as in Fig. 1. Data are the mean of triplicate wells ± SD and are representative of two separate experiments.

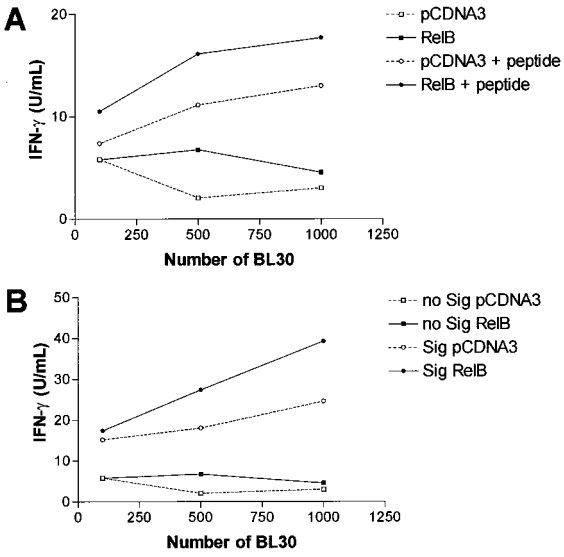

Fig. 3. RelB enhances MHC class I-restricted peptide presentation to a CD8$^+$ CTL line. The CTL line LC13 is specific for the peptide sequence FLRGRAYGL, restricted by HLA-B8. (*A*) BL30 were transfected with either RelB or pCDNA3 and incubated for 24 h. Nonviable cells were removed by density-gradient centrifugation, and varying numbers of cells from each transfected population were added to 10^4 LC13 in the presence or absence of 1 μg peptide. hIFN-γ levels were measured in the supernatant by ELISA after 24 h. (*B*) As above, 24 h after transfection, varying numbers of RelB or pCDNA3-transfected BL30 stably expressing FLRGRAYGL with or without endoplasmic reticulum signal sequence were added to 10^4 LC13, and hIFN-γ was measured after 24 h. Data are the mean of triplicate wells and are representative of three separate experiments.

amounts of RelB were detectable in nuclear extracts derived from RelB-transfected BJAB (Fig. 2*A*, lane 6).

By immunohistochemical staining, the transfection efficiency of BJAB or BL30 was 10%–20%. To enrich RelB$^+$ cells, BJAB were cotransfected with the GFP expression vector pGreenLantern (detectable flow cytometrically on the FITC channel) and either RelB or pCDNA3. FITC$^+$ cells were sorted by flow cytometry 24 h later, cytospun, and stained with RelB antibody. Seventy to eighty percent of transfected, sorted BJAB had located large amounts of RelB to the nucleus (Fig. 2*B*). Similar data were obtained for the BL30 cell line (data not shown). To test the effect of transcriptionally active nuclear RelB on antigen-presenting capacity, BJAB or BL30 cells sorted in this manner were used as stimulators in allogeneic MLR. Cells transfected with RelB were more effective APC than cells transfected with pCDNA3 (Fig. 2*C*). Taken together, these data demonstrate that RelB transfected into BL lines is located to the nucleus, is potentially transcriptionally active, and enhances the capacity of the cells to stimulate proliferation of resting allogeneic T cells.

Because the allogeneic T cell response results from presentation predominantly of self antigens, the next experiments examined whether RelB overexpression would also enhance specific peptide presentation in an MHC class I-restricted response. The cell line BL30 is a target for the CTL line, LC13, in the presence of the B8-restricted EBNA3 peptide FLRGRAYGL. Peptide presentation is associated with IFN-γ production by LC13. Transfection of BL30 with RelB enhanced the capacity of this line to present HLA-B8-restricted peptide to LC13 even in the absence of enrichment of nuclear RelB$^+$ cells by cell sorting (Fig. 3*A*).

To determine whether RelB transcriptional activity could also augment presentation of an antigen processed endogenously, plasmids encoding the same EBNA3 peptide with or without an endoplasmic reticulum targeting signal sequence were used (Fig. 3*B*). Whereas RelB enhanced presentation of endogenously processed signal sequence tagged peptide, there was no enhancement in the absence of the signal sequence tag. The data indicate that RelB augments antigen presentation of exogenous MHC class I-restricted peptide and endogenously processed antigen. However, up-regulation is not associated with augmentation of TAP gene expression. Furthermore, no up-regulation of TAP occurred in RelB-transfected cells (data not shown). Rather, up-regulation of MHC or costimulatory molecule expression is more likely.

Effect of RelB Expression on Cell Surface Markers. Because ectopic expression of RelB was able to enhance the ability of BL cell lines to act as APC, the next experiments examined the effect of RelB

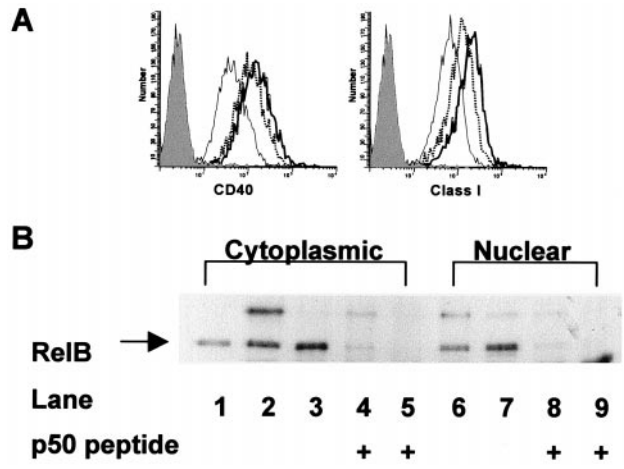

Fig. 4. Effects of CD40 ligation and ectopic expression of RelB on BJAB cell surface markers. (*A*) BJAB were transfected with 10 μg RelB (dotted line), 10 μg RelB, and 10 μg p50 (bold line) or 10 μg pCDNA3 (thin line). In each case, BJAB were cotransfected with 5 μg pGreenLantern and incubated for 24 h before staining with PE-conjugated anti-CD40 or anti-MHC class I. Cells were analyzed by two-color flow cytometry, gating on GFP^+ cells. Isotype controls are indicated by the filled histograms. Significant differences were observed in levels of CD40 (see Supplementary Fig. 7*A* at www.pnas.org) ($P = 0.0212$) and MHC class I (see Supplementary Fig. 7*B* at www.pnas.org) ($P = 0.0107$) after transfection of RelB in BJAB. Data are representative of three separate experiments. (*B*) Cytoplasmic or nuclear extracts from BJAB transfected with RelB (lanes 3 and 7) or pCDNA3 (lanes 2 and 6) were immunoprecipitated after 24 h with anti-p50, and immunoblots were probed with anti-RelB. p50-blocking peptide was included in the immunoprecipitation of extracts of RelB- (lanes 5 and 9) or pCDNA3- (lanes 4 and 8) transfected BJAB. Lane 1 indicates constitutive RelB present in BJAB cytoplasmic extracts.

overexpression on surface molecules important for APC function. BJAB and BL30 were cotransfected with RelB/GFP or pCDNA3/GFP and stained 4, 8, and 24 h later with various PE-labeled antibodies directed against MHC, adhesion, and costimulatory molecules. For both RelB- and pCDNA3-transfected cells, GFP^+ cells were gated to allow analysis and comparison of PE expression. No change in the levels of cell surface HLA-DR, CD95, CD54 [intracellular adhesion molecule 1 (ICAM-1)], CD106 [vascular cell adhesion molecule 1 (VCAM-1)], CD58 [leukocyte function-associated antigen 3 (LFA-3)], CD80, or CD86 were observed (data not shown). In contrast, overexpression of RelB was associated with up-regulation of cell surface CD40 and MHC class I, which was maximal at 24 h (dotted line, Fig. 4*A*; $P < 0.05$). Furthermore, whereas transfection of either p50, p52, or RelB/p52 had no effect on CD40 and MHC class I expression (data not shown), cotransfection of p50 and RelB further increased surface expression of CD40 and MHC class I relative to transfection with RelB alone (bold line, Fig. 4*A*; $P < 0.05$). The increased expression suggests that p50 and RelB heterodimerize for transcriptional effects on CD40 and MHC class I. Furthermore, in the absence of p50 overexpression, transfection of RelB is likely to heterodimerize with available constitutive p50 (Fig. 1*B*). It should be noted, however that both CD40 and MHC class I are constitutive in B cells, including BL cell lines, and that RelB up-regulates rather than switches on expression, with functional consequences for its role as an APC. To determine whether nuclear RelB was coassociated with p50 after RelB overexpression in BJAB, p50 or p52 were immunoprecipitated from nuclear and cytoplasmic extracts, and immunoreactive RelB was analyzed (Fig. 4*B*). Whereas levels of cytoplasmic p50-associated RelB were similar in pCDNA3- (Fig. 4*B*, lane 2) and RelB- (Fig. 4*B*, lane 3) transfected BJAB, nuclear p50-associated RelB was increased in RelB-transfected BJAB (Fig. 4*B*, lane 7) relative to pCDNA3 transfected BJAB (Fig. 4*B*, lane 6). There was a 3-fold increase measured by densitometry. However, because these experiments were carried out in the absence of $RelB^+$ cell enrichment by cell sorting, the increase in nuclear RelB in transfected cells was likely to be higher. A higher molecular weight RelB complex was also observed in pCDNA3-transfected BJAB (Fig. 4*B*, lanes 2 and 6) and may represent inactive p105/RelB complex. In contrast, after immunoprecipitation of nuclear extracts with anti-p52, no immunoreactive RelB could be detected (data not shown).

In summary, the data demonstrate that overexpression of RelB is associated with up-regulation of MHC class I and CD40 molecule expression and CD40 protein synthesis in BL cell lines. Because p50 but not p52 coexpression augments MHC class I and CD40 induction and is found to associate with RelB in the nucleus, the p50/RelB complex is likely to mediate these effects.

Antisense RelB Diminishes Antigen-Presenting Capacity of BL Lines. The APC function of the BL cell line, DG75, is intermediate between LCL and BJAB lines (data not shown). EMSA and supershift analysis demonstrated constitutive and inducible RelB and p50 in nuclear extracts of DG75 (Fig. 5*A*). To down-regulate constitutive RelB, a full-length antisense-RelB sequence was cloned into pCDNA3. Either this construct or control pCDNA3 was transfected into DG75, and stable bulk populations were generated. The efficacy of antisense-RelB was assessed by incubating the stable lines in sCD40L for 4 or 24 h, followed by RelB immunohistochemical staining. Control DG75 expressed predominantly cytoplasmic RelB (Fig. 5*B*). After 4 h culture with sCD40L, strong nuclear RelB staining was observed in approximately 20% of cells and weak nuclear staining in about 50% of cells (Fig. 5*B*). After 24 h, RelB was predominantly cytoplasmic, and approximately 30% of cells contained weak nuclear RelB (data not shown). In contrast, translocation of RelB to the nucleus in antisense-RelB DG75 was reduced constitutively, and sCD40L treatment had little effect (Fig. 5*B*). Thus, antisense-RelB effectively reduced, albeit not completely, the expression of RelB in stably transfected DG75.

The effect of antisense-RelB on cell surface molecule expression was examined by culture of DG75 lines for 24 h in the presence of sCD40L. Whereas antisense-RelB had no effect on the levels of CD80, CD86 (data not shown), the up-regulation of MHC class I and II (Fig. 5*C*) and, to a lesser extent, CD54 expression (data not shown) induced by sCD40L was blocked by antisense-RelB. There was no detectable effect of sCD40L on CD40 expression by wild-type DG75, most likely because of blockade of the antibody-binding epitope by sCD40L. The capacity of antisense-RelB DG75 to stimulate the allogeneic MLR was reduced, compared with pCDNA3 DG75 (Fig. 5*D*). Furthermore, 24 h preincubation with sCD40L was unable to completely restore the APC function of antisense-RelB DG75. These data confirm that RelB transcriptional activity affects APC function through several key surface molecules.

Discussion

NF-κB/Rel family members are important transcription factors for the immune response. In recent years, studies of mice either overexpressing or bearing various mutations of individual Rel family members have constructed a more complete picture of the specific roles of RelA, c-Rel, p50, p52, and RelB in the generation of immune responses. $RelB^{-/-}$ mice manifest a complex phenotype comprising myeloid infiltration of the liver and spleen, defective immune responses, and an atrophic thymic medulla and absent lymph nodes (8, 10). Whereas the explanation for all these defects (some of which may be counterregulatory) is not known, it is clear that these animals display deficient negative selection and an enlarged pool of self-reactive

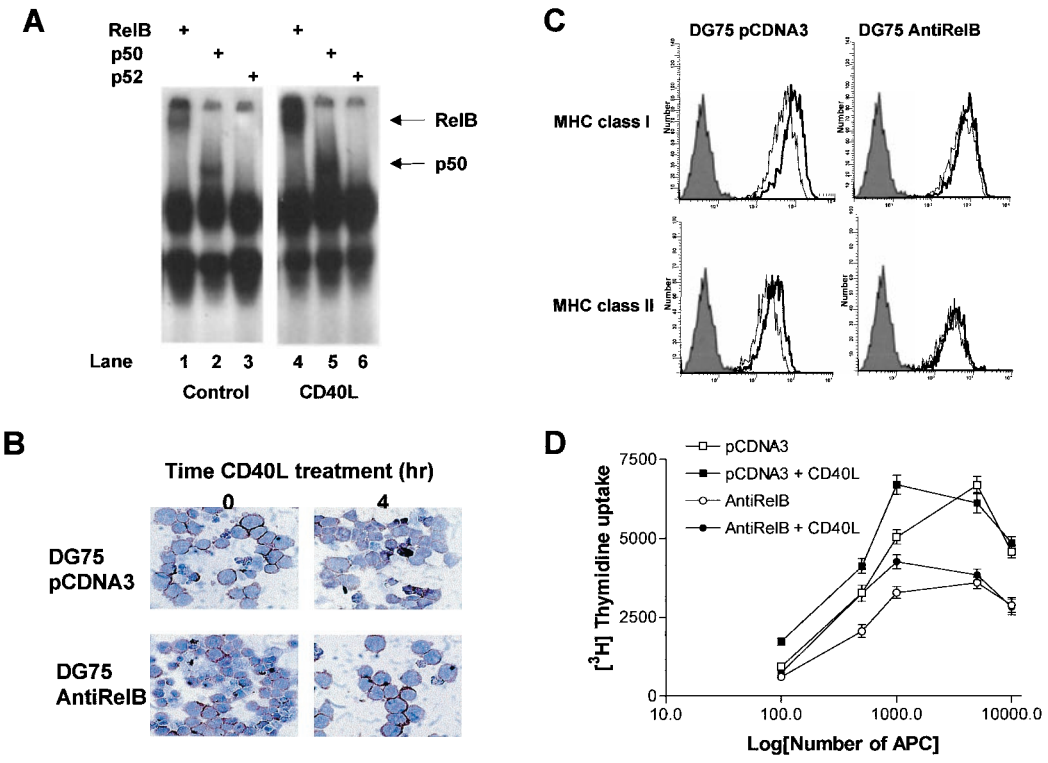

Fig. 5. RelB down-regulation leads to diminished APC function. (*A*) EMSA and supershift analysis of nuclear extracts prepared from DG75 incubated for 24 h in the presence or absence of 50 ng/ml sCD40L. (*B*) DG75 lines stably transfected with pAntiRelB or pCDNA3 were incubated with or without sCD40L for 0 or 4 h, then cytospun. Slides were fixed in paraformaldehyde and stained with anti-RelB by using diaminobenzidine as the chromogen (black, magnification ×40). (*C*) pAntiRelB or pCDNA3 DG75 lines were incubated in the presence (bold line) or absence (thin line) of 50 ng/ml sCD40L, then stained for expression of MHC class I and MHC class II. Isotype controls are indicated by the filled histograms. Significant differences were observed in levels of MHC class I (see Supplementary Fig. 7*C* at www.pnas.org) ($P = 0.0202$) and MHC class II (see Supplementary Fig. 7*D* at www.pnas.org) ($P = 0.0463$) after CD40L treatment of pCDNA3-DG75 but not for pAntiRelB-DG75. Data are representative of three separate experiments. (*D*) Stably transfected pAntiRelB- or pCDNA3-transfected DG75 were incubated in the presence or absence of 50 ng/ml sCD40L for 24 h, washed, and used in MLR as described. Data are the mean of triplicate wells ± SD and are representative of three separate experiments.

lymphocytes. In addition, the inhibition of proinflammatory signals to fibroblasts is deficient, and the distribution of myeloid-related DC is abnormal, likely as a result of a lack of differentiation (11, 21, 22). In contrast, lymphoid-related DC populate the lymphoid organs of these animals in normal numbers (11). The function of DC from $RelB^{-/-}$ mice has been examined in several ways. DC differentiated *in vitro* in the presence of granulocyte-macrophage colony-stimulating factor from $RelB^{-/-}$ bone marrow demonstrated reduced APC function and clustering capacity compared with DC differentiated from wild-type marrow (9). *In vivo*, the animals displayed a reduced delayed type hypersensitivity response (8). The APC function of $RelB^{-/-}$ B cells has not specifically been addressed. However, proliferation and IgG production were reduced in $RelB^{-/-}$ B cells. This defect in Ig production was much less profound than in $cRel^{-/-}$ B cells (23). Human circulating DC precursors are RelB negative, and DC differentiation is associated with RelB synthesis and nuclear location, as well as acquisition of potent APC function (12). Based on the implication of RelB in APC function in mice, and the specific location of RelB to the nuclei of APC in human tissue *in vivo*, the effect of RelB expression and inhibition on APC function was addressed.

The data demonstrate that RelB transcriptional activity can directly affect antigen presentation of BL cell lines, and that this is likely to be mediated by at least two known cell surface molecules, CD40 and MHC class I. Whereas the functional role of RelB in normal cells, particularly DC, is of great interest, transformed BL cell lines were examined in the current study because they are relatively homogeneous and are readily transfected both transiently and stably. On the other hand, DC develop from heterogeneous precursors, some of which spontaneously up-regulate RelB expression. For example, purified human peripheral blood myeloid DC precursors are $RelB^{-}$, but express and translocate RelB within several hours of incubation (ref. 12 and unpublished data). Peripheral blood monocyte-derived DC populations contain predominantly cytoplasmic RelB and variable proportions of cells with nuclear RelB (12). Furthermore, neither population proliferates, precluding stable transfection (16). The current studies demonstrate that transient overexpression of RelB that induces location to the nucleus has two effects in BL cell lines. First, expression of CD40 and MHC class I surface molecules is increased above the constitutive levels of expression of these molecules. Second, nuclear $RelB^{+}$ BL lines stimulate resting T cells in the MLR more effectively, and their ability to present MHC class I-restricted peptide to specific T cells is enhanced.

It is of interest that the effects of RelB on MHC class I, CD40, and APC function are relatively subtle. Thus, RelB appears to confer a qualitative effect to B cell APC function, as opposed to a major genetic switch. These results are not unexpected, in view of the qualitative changes occurring in DC on transcription and translocation of RelB to the nucleus. These events are associated with "maturation" of the DC, which also entails up-regulation of expression of a number of molecules, including MHC molecules and CD40, as well as an enhanced capacity for antigen presentation (12, 24).

The effects of transient RelB transfection were not observed in stable RelB-transfected BL cell lines. All stable lines generated expressed increased amounts of cytoplasmic but not nuclear RelB (data not shown), indicative of homeostatic mechanisms for regulation of RelB within the cell by IκB. In this regard, RelB has been shown to up-regulate IκBα itself (25), thus suggesting a negative feedback loop. It has been previously shown that the IκB inhibitors, p100 and p105, were strongly associated with RelB after immunoprecipitation in Daudi cell extracts (26) and that addition of increasing amounts of IκBα can inhibit p50/RelB and p52/RelB binding (27). Furthermore, cotransfection experiments in Jurkat cells indicate that IκBα overexpression represses both p50/RelB and p52/RelB κB-reporter induction (27). Therefore, the data indicate that both p100 (IκBδ) and IκBα can bind and inhibit p50/RelB and p52/RelB heterodimers.

Activation of NF-κB in B cells is mediated by inflammatory cytokines, including TNFα and IL-1α, bacterial or viral stimuli and CD40L (28). CD40 is a member of the TNF receptor (TNFR) superfamily (29). CD40L, expressed by activated $CD4^+$ T cells, provides signals for B cell function and induces nuclear translocation of RelB and RelA in conjunction with p50 (4). Ligation of CD40 on DC up-regulates expression of the costimulatory molecules CD80 and CD86 and enhances IL-12 production (30). The up-regulation of CD40 by RelB/p50, as shown here, potentially provides a powerful amplification response between APC and CD40L expressed by T cells. Thus, it is possible that RelB plays a particular role in "fine-tuning" the APC in response to T cell cross-talk.

RelB is unable to form homodimers and has shown to be transcriptionally active when dimerized with p50 or p52 (2, 3, 31). Several pieces of evidence indicate that p50, not p52, partners RelB in its transcriptional role in APC function. First, cotransfection with p50 but not p52 enhanced the effects of RelB transfection on cell surface molecule expression. Second, immunoprecipitation of p50 but not p52 revealed coassociation of RelB in the nucleus of RelB-transfected BL cells. Third, activation of the MHC class I NF-κB promoter by RelB/p50 heterodimers in reporter assays has been previously shown (6). These results are consistent with the immune defects of p50-deficient mice and increased severity of the defects in RelB/p50 double knockout mice, as opposed to a specific defect in B cell follicle and germinal center formation of p52 mutant mice (32–34).

Previously, by using fibroblasts derived from RelB deficient mice, RelB has been shown to play a regulatory role in the transcription of proinflammatory chemokines (22). Lack of regulatory activity is likely to explain, in part, the inflammatory phenotype of RelB mutant animals. In contrast, the current studies demonstrate enhancement of antigen-presenting capacity by RelB transcriptional activity. In a similar fashion, both stimulatory and inhibitory effects of the p105 precursor of p50 have been demonstrated, depending on the cell type (35). It is also possible that counterregulatory mechanisms, particularly occurring during the bone marrow culture required for DC generation, led to different effects in the mutant mice to those afforded by transient transfection, as assessed in the current studies.

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